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13. ABSTRACT (Maximum 200 Words)

Circadian clock disruption may be correlated to increased risk of breast cancer in humans and has been linked to tumor progression and development in mice. We tested mice and cell lines lacking both the Cryptochrome1 and Cryptochrome2 circadian clock genes, rendering them arrhythmic. We find that arrhythmic Cryptochrome knockout mice and cells, respectively, are indistinguishable from wild-type controls with regard to ionizing radiation-induced morbidity and mortality and cell cycle checkpoint response, respectively. We confirm upregulation of the anti-mitotic kinase Wee1 as has been previously reported in Cryptochrome knockout liver tissue; however, there does not appear to be a functional consequence of this upregulation with regards to cell cycle checkpoint response. We find that c-Myc, which was reported to be upregulated in the absence of circadian gene Period2, is unaffected by loss of Cryptochrome. We are currently investigating expression of other cell-cycle related genes in Cryptochrome knockout mouse tissues and cells. Our current results indicate that circadian clock disruption per se does not predispose mice to cancer; rather, the effects previously reported are likely due to contributions of individual clock proteins to damage response pathways.

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INTRODUCTION

The circadian clock and the cell cycle are two global oscillatory systems in most eukaryotes which are responsible for regulation of many essential pathways and processes. It has long been suspected that these two systems, both of which oscillate with a periodicity of approximately 24 hours, are somehow connected but the nature of this relationship has not been well-defined. Reports of epidemiological studies that circadian rhythm disruption due to shift work increases breast cancer risk in women (Hansen, 2001) indicate that there is indeed a link between the cell cycle and the circadian clock. More recent reports show that genetic or surgical disruption of the clock can cause increased rates of tumor growth in mice (Fu *et al*, 2002; Filipski *et al*, 2002). The goal of this work is to investigate the effect of genetic circadian clock disruption on tumorigenesis and the cell cycle in a mouse system using mice deficient in *Cryptochrome1* and *Cryptochrome2*, two of the core mammalian circadian clock genes.

The specific aims of this proposal are as follows:

1. **We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.**

2. **We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.**

3. **We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.**

Specific Aim 1: We will determine the effect of circadian clock disruption on uninduced tumor development in cryptochrome-deficient mice.

We set aside a small population (11 of each genotype) of wild-type mice and *Cryptochrome1$^{-/-}$ Cryptochrome2$^{-/-}$* mice (*Cry1$^{-/-}$Cry2$^{-/-}$*, Selby *et al*, 2000; Vitaterna *et al*, 1999) for observation of tumor development under normal, uninduced conditions. Due to the inefficient breeding we observe for *Cry1$^{-/-}$ Cry2$^{-/-}$* mice, the cohort of mice used for this study was relatively small. We find that unperturbed wild-type mice die with a frequency similar to that of *Cry1$^{-/-}$Cry2$^{-/-}$* mice (data not shown; Gauger and Sancar, 2005).

Specific Aim 2: We will determine the susceptibility of mice with disrupted circadian rhythms to ionizing radiation.

We have set aside a population of wild-type mice (24) and *Cry1$^{-/-}$Cry2$^{-/-}$* mice (27) for observation of susceptibility to ionizing radiation (IR). Mice were treated with a single dose of 4 Gy of IR at approximately 8 weeks of age. Treatment was done at zeitgeiber time (ZT) 10, which has been reported to be the time of day at which the mitotic index in murine bone marrow is highest (Bjarnason and Jordan, 2000). Mice were observed for 95+ weeks after treatment for morbidity and mortality and

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the results were analyzed using the Kaplan-Meier method of determining survival rate (Figure 1; Gauger and Sancar, 2005). Our results show that wild-type mice and $Cry1^{-/-}Cry2^{-/-}$ mice show similar survival rates after treatment with IR. Moreover, no overt tumors were observed in mice of either genotype over the course of the study or as causes of death. Irradiated mice died from a variety of causes including paralysis, seizures, and genitourinary prolapses and infection that necessitated euthanasia, and in some cases indeterminable causes.
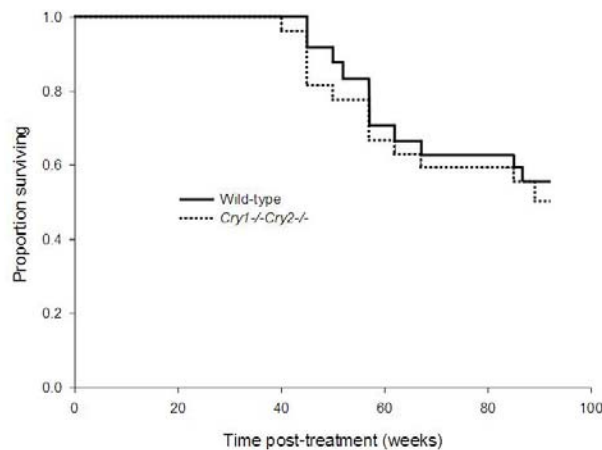
Figure 1: Crytpochrome-deficient mice do not show increased sensitivity to ionizing radiation. Mice irradiated at 8 weeks of age with a single dose of 4 Gy of IR were observed over 95+ weeks for morbidity and mortality. Survival is plotted using the Kaplan-Meier method. (Gauger and Sancar, 2005)

(Note: Originally we intended to repeat this analysis using mice in a retinal degeneration background to confirm that any change in IR response that we observed was indeed due to the circadian phenotype of the animals. However, because our data indicates no change in IR response between wild-type and $Cry1^{-/-}Cry2^{-/-}$ mice, we elected not to perform the analysis in an alternative background.)

Specific Aim 3: We will use cell-based assays to investigate the cell cycle phenotype of cryptochrome-deficient cells under normal and induced conditions.

For this set of experiments we have used wild-type and $Cry1^{-/-}Cry2^{-/-}$ fibroblast cell lines isolated from our animals (Thompson et al, 2004). Both cell lines underwent spontaneous immortalization. Our goal was to determine the cell cycle phenotype of both cell lines with respect to cell cycle arrest after DNA damage and DNA repair after damage.

We used flow cytometry to determine cellular response to IR. Cells were treated with 8 Gy of IR and DNA content analysis was performed at the indicated timepoints (Table 1). Percentage of cells in G1, S, and G2/M phases of the cell cycle was quantitated and is shown in Table 1. Our data shows that both wild-type and $Cry1^{-/-}Cry2^{-/-}$ fibroblasts underwent a G2/M arrest as a result of DNA damage by IR. Moreover, both cell types recovered from the G2/M arrest with similar kinetics.

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Genotype	Time (h) post-IR (8 Gy)	% cell population		
		G1	S	G2/M
Wild-type	0	45.16	30.28	**25.98**
	4	31.53	36.24	34.08
	8	14.25	31.41	**56.06**
	12	18.09	16.85	65.72
	24	39.71	27.95	**33.68**
$Cry1^{-/-}Cry2^{-/-}$	0	41.20	34.34	**26.35**
	4	24.17	42.43	36.37
	8	10.64	36.63	**56.50**
	12	21.04	9.91	68.05
	24	39.08	26.88	**32.94**

Table 1: Cellular arrest in response to IR. Cells of the indicated genotypes were treated with 8 Gy of IR and DNA content analysis was performed at the indicated timepoints. (Gauger and Sancar, 2005)

To analyze DNA repair after treatment with DNA damaging agents, clonogenic assays were performed. Cells were plated at low density and treated with the indicated doses of either ultraviolet light (UV) or IR. After growing for 9-10 days, colonies were stained and counted. As Figures 2A and 2B show, both wild-type and $Cry1^{-/-}Cry2^{-/-}$ fibroblasts show identical survival profiles after treatment with either UV or IR. These data indicate that the DNA damage checkpoint and DNA repair pathways are operating normally in both cell types and that $Cry1^{-/-}Cry2^{-/-}$ fibroblasts are not deficient in either of these pathways.
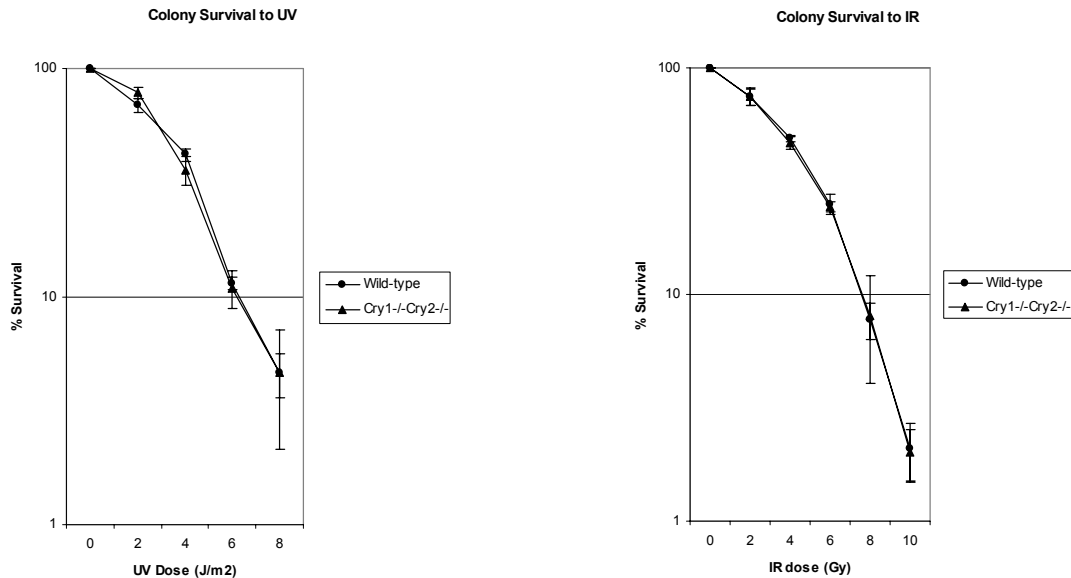
Figure 2: Cellular survival in response to UV or IR. (A) Colony survival after treatment with UV. (B) Colony survival after treatment with IR. Cells were treated with the indicated doses of either UV or IR and allowed to form colonies for 9-10 days. Colonies were fixed and stained with Giemsa stained and survival is plotted. (Gauger and Sancar, 2005)

In addition to analyzing the DNA damage checkpoint response in cultured wild-type and $Cry1^{-/-}$ $Cry2^{-/-}$ fibroblasts, we have also analyzed expression profiles of proteins involved in cell cycle control. Wee1, an anti-mitotic kinase which inhibits cells from entering mitosis, has been reported to be

upregulated in *Cry1⁻/⁻Cry2⁻/⁻* mouse livers (Matsuo et al, 2003). The oncogene *c-Myc* has been reported to be upregulated in the absence of *Period2* in mouse livers (Fu et al, 2002). We analyzed expression of both Wee1 and c-Myc proteins in wild-type and *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue and fibroblasts. As shown in Figure 3A-B, c-Myc protein expression in *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue (A) and fibroblasts (B) is indistinguishable from that of wild-type (Gauger and Sancar, 2005).
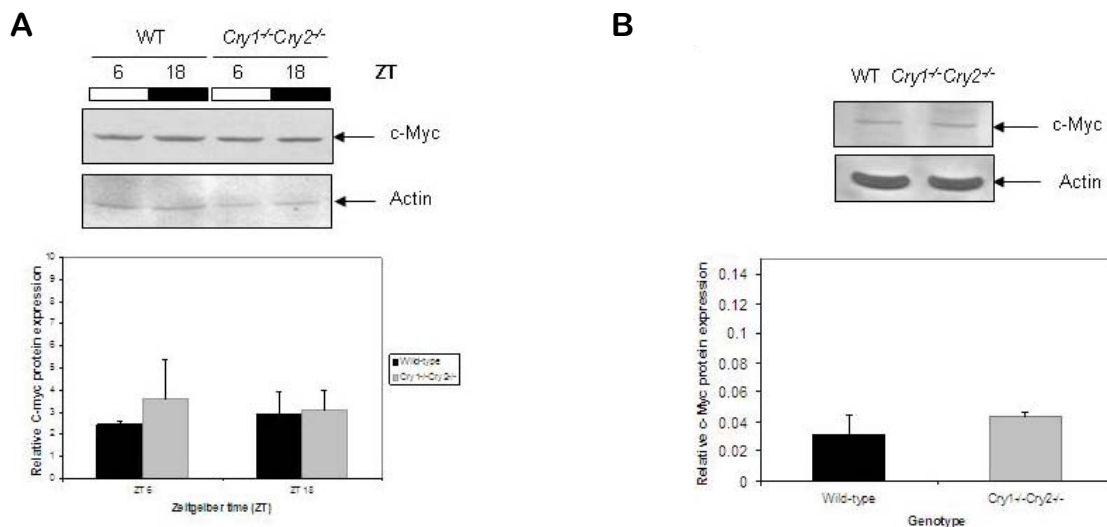
Figure 3: Western blotting of wild-type and *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue (A) and fibroblasts (B) for c-Myc expression. Quantitation is shown below Western blots. Actin is used as a loading control. (Gauger and Sancar, 2005)

We also examined Wee1 protein expression in *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue and fibroblasts, to confirm earlier reports. We find that, as shown in Figure 4A-B, Wee1 protein levels are indeed upregulated in *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue and fibroblasts relative to wild-type (Gauger and Sancar, 2005). However, our DNA damage response data (Figures 1 and 2, Table 1) indicate that this upregulation does not have a functional consequence in the assays we used, nor does it cause an observable change in cellular growth rate in culture (Figure 4C).
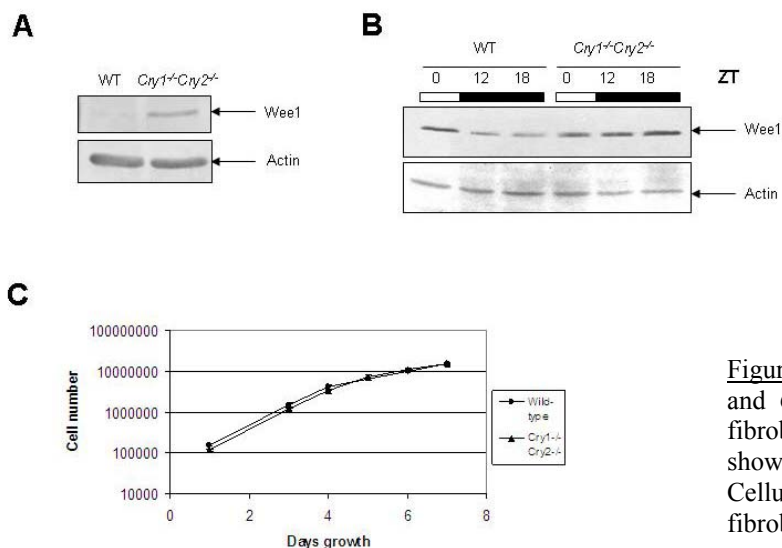
Figure 4: (A and B) Wee1 status in wild-type and *Cry1⁻/⁻Cry2⁻/⁻* mouse liver tissue (A) and fibroblasts (B). Western blotting for Wee1 is shown. Actin is used as a loading control. (C) Cellular growth of *Cry1⁻/⁻Cry2⁻/⁻* mouse fibroblasts.

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KEY RESEARCH ACCOMPLISHMENTS

- Mice with genetic disruption of the circadian clock through loss of core circadian clock genes *Cryptochrome1* and *Cryptochrome2* do not show increased morbidity or mortality relative to wild-type mice. **(Specific Aim 1)**
- Mice with genetic disruption of the circadian clock through loss of *Cryptochrome1* and *Cryptochrome2* do not show increased sensitivity to IR or increased susceptibility to IR-induced tumorigenesis, relative to wild-type mice. **(Specific Aim 2)**
- Both wild-type and Cryptochrome-deficient fibroblasts show similar responses to DNA damaging agents with respect to DNA damage checkpoint-mediated cell cycle arrest. **(Specific Aim 3)**
- Both wild-type and Cryptochrome-deficient fibroblasts show normal abilities to repair DNA in response to DNA damage by either UV or IR. **(Specific Aim 3)**
- Wee1 protein levels are upregulated in the absence of Cryptochromes, and c-Myc protein levels are unaffected in the absence of Cryptochromes. **(Specific Aim 3)**

REPORTABLE OUTCOMES

- ABSTRACT
  Gauger MA and Sancar A. Cryptochrome, circadian clock, cell cycle, and cancer. *Keystone Symposia 2005 Cancer and Development Abstract Book* 2005: 121.

- PRESENTATION
  Gauger MA. Cryptochrome, circadian clock, cell cycle, and cancer. Keystone Symposium 2005: Cancer and Development. February 5-February 10, 2005, Banff, Alberta, Canada.

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- PUBLICATION
  Gauger MA and Sancar A. (2005) Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Research*, **65(15)**: 6828-34.

CONCLUSIONS

Our current progress on the work outlined in our proposal leads us to conclude that circadian clock disruption per se does not predispose mice to cancer. It has been shown that disruption of a core circadian clock gene, *mPeriod2*, results in increased tumorigenesis and cell cycle disruption in mice; however, this phenomenon is not seen after disruption of *Cryptochrome1* and *Cryptochrome2* and thus is not an absolute consequence of all types of circadian clock disruption. It may be that individual clock genes also participate in other pathways regulating cell cycle and this accounts for the reported

phenomena in *mPeriod2^{m/m}* mice. It will be interesting to continue this work and determine the expression profiles of a panel of cell cycle control genes in circadian clock-deficient systems. This work will help to discover the mechanism by which some types of circadian clock disruption can result in increased tumor predisposition.

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Cryptochrome, Circadian Cycle, Cell Cycle Checkpoints, and Cancer

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Abstract

It has been reported that disruption of the circadian clock may lead to increased risk of breast cancer in humans and to a high rate of ionizing radiation–induced tumors and mortality in mice. Cryptochrome 1 and cryptochrome 2 proteins are core components of the mammalian circadian clock and mice mutated in both genes are arrhythmic. We tested $Cry1^{-/-}$ $Cry2^{-/-}$ mice and fibroblasts derived from these mice for radiation-induced cancer and killing and DNA damage checkpoints and killing, respectively. We find that the mutant mice are indistinguishable from the wild-type controls with respect to radiation-induced morbidity and mortality. Similarly, the $Cry1^{-/-}$ $Cry2^{-/-}$ mutant fibroblasts are indistinguishable from the wild-type controls with respect to their sensitivity to ionizing radiation and UV radiation and ionizing radiation–induced DNA damage checkpoint response. Our data suggest that disruption of the circadian clock in itself does not compromise mammalian DNA repair and DNA damage checkpoints and does not predispose mice to spontaneous and ionizing radiation–induced cancers. We conclude that the effect of circadian clock disruption on cellular response to DNA damage and cancer predisposition in mice may depend on the mechanism by which the clock is disrupted. (Cancer Res 2005; 65(15): 6828-34)

Introduction

The circadian clock and cell cycle are two global regulatory systems in most eukaryotic organisms. It has been known for some time that disruption of the circadian rhythm by genetic or environmental factors causes a variety of disorders in humans, such as sleep disturbances, seasonal affective disorder, and jet lag. Disruption of cell cycle regulation causes cancer. Recent epidemiologic studies have raised the possibility that disruption of the circadian clock may also increase cancer risk in humans (1) and adversely affect prognosis in cancer patients (2). In particular, it was reported that women working nightshift exhibited a significant increase in breast cancer risk (3, 4). Similarly, it was reported that cancer patients with altered circadian rhythm had poorer survival relative to patients with normal rhythm (5).

These epidemiologic studies were complemented by studies with mouse model systems. In one study, transplantation of an osteosarcoma or a pancreatic adenocarcinoma into mice with ablations to the master circadian clock, the suprachiasmatic nuclei, caused accelerated tumor growth rate relative to animals with intact suprachiasmatic nuclei (6). In a second study, it was found that mice that were rendered arrhythmic by repeat 8-hour advance of the light-dark cycle every 2 days exhibited faster rates of implanted tumor growth relative to control mice maintained under a light/dark 12-hour/12-hour cycle (7).

Finally, the circadian rhythm was disrupted in mice by targeted mutations of the core clock genes that engender the molecular clock not only in the suprachiasmatic nuclei but in all peripheral organs and the effects of this disruption on cell growth and spontaneous and ionizing radiation–induced tumor incidence were analyzed. The core clock proteins are Clock and Bmal1 that act as transcriptional activators of the *Cryptochrome* (*Cry*), *Period* (*Per*), and *Bmal1* genes and the Cry1, Cry2, Per1, and Per2 proteins that function as transcriptional repressors of the Clock-Bmal1-driven genes (refs. 8, 9; Fig. 1). The effect of these core clock proteins is modulated by additional proteins such as Rev-Erbα and CK1ε to generate a rather precise molecular oscillator with ~24-hour periodicity. This periodicity is transmitted to the clock-controlled genes (CCG) that constitute about 10% of the expressed genes in a given tissue to generate rhythmic outputs at the physiologic and behavioral levels (10). The molecular mechanism of the mammalian circadian clock has been elucidated in considerable detail in recent years, making it possible to investigate the interfacing of this global regulatory pathway with other global regulatory systems, such as cell cycle checkpoints, at a mechanistic level. One such study found that in mice with a Per2 mutation, *c-Myc* transcription was up-regulated and p53 was down-regulated; consequently, these animals had increased incidence of spontaneous and ionizing radiation–induced lymphomas and an increased rate of mortality after ionizing radiation (11). Another study reported that in crypto-chromeless mice, Wee1 antimitotic kinase was elevated and, consequently, liver regeneration in these mice following partial hepatectomy was delayed relative to wild-type controls (12). Finally, we have recently found that the mammalian Timeless protein, which is considered to be a clock protein according to some studies (13) but not others (14), binds to DNA damage checkpoint proteins ATR and Chk1 and is essential for the DNA damage checkpoint response (15).

Taken together, the epidemiologic data, the data from mouse model systems, and that from cell-based assays have led to an emerging consensus that the circadian cycle and cell cycle are tightly coupled and that disruption of the circadian cycle by any means would disrupt cell cycle checkpoints as well, causing animals with circadian clock disruption to be more prone to spontaneous and DNA damage-induced cancers (1–4). Within this conceptual framework, then, we wished to examine the effect of circadian clock disruption caused by cryptochrome knockout on DNA damage checkpoints and on predisposition to spontaneous and ionizing radiation–induced cancers. We find that clock disruption by cryptochrome knockout does not measurably affect DNA damage checkpoints and does not cause mice to be more susceptible to cancer. We conclude that circadian clock disruption

Figure 1. Model for circadian clock in mammals. Clock and Bmal1 are transcriptional activators that make up the Clock-Bmal1 complex, which activates the transcription of *Cry*, *Per*, and *Bmal1* genes. The Cry (Cry1 and Cry2) and Per (Per1 and Per2) proteins are transcriptional repressors that interfere with the activity of Clock-Bmal1 and down-regulate transcription of genes controlled by these factors. In addition, Per and Cry proteins stimulate the transcription of *Bmal1* by an ill-defined mechanism. CCGs, which include *Wee1* and *c-myc*, are regulated by the clock proteins but do not affect the activity of the clock proteins or the transcription of the clock genes. Wee1 expression is positively regulated by Clock-Bmal1, and c-Myc expression is repressed by this complex. Hence, in the absence of Cry or Per, Wee1 expression is expected to be upregulated due to lack of inhibition of Clock-Bmal1 by Cry and Per. It has been reported that *c-myc* transcription is elevated in *Per2* mutant mice because of a reduced level of Bmal1, which represses the *c-myc* promoter.
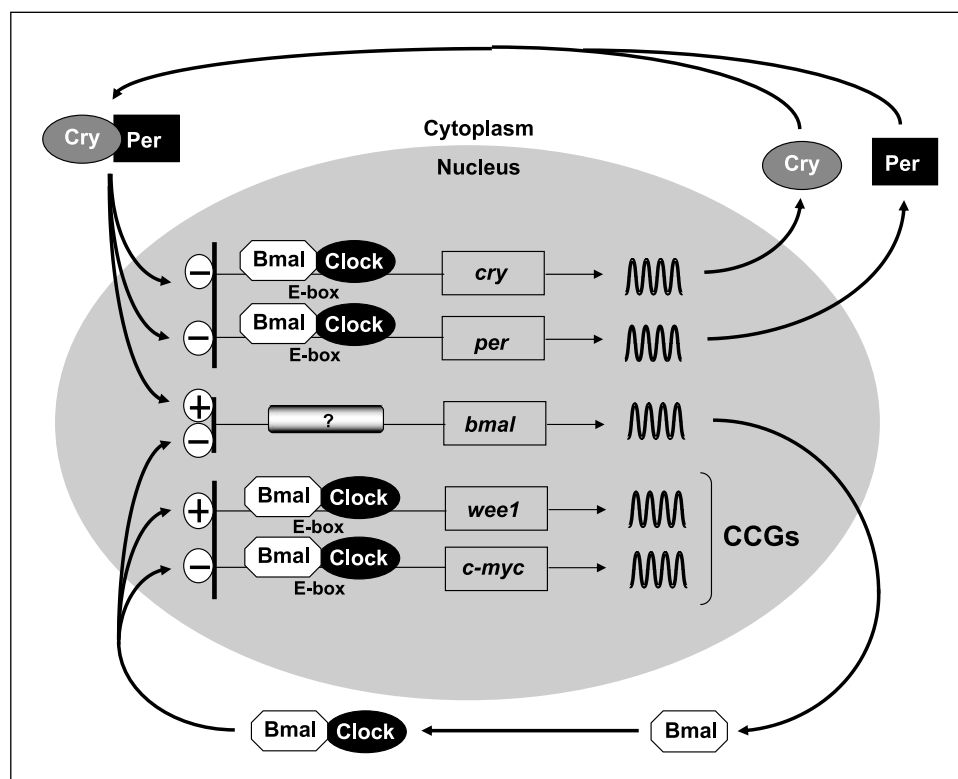
per se does not prime cells to cancerous transformation, presumably due to the presence of homeostatic mechanisms, in addition to the circadian rhythm, that regulate cell cycle and cellular responses to DNA damage.

Materials and Methods

Mice, ionizing radiation treatment, and survival. $Cry1^{-/-}Cry2^{-/-}$ mice (16, 17) were backcrossed with C57BL/6J six times to obtain Cry mutant mice in essentially the same genetic background as wild-type control C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were maintained on a light/dark 12:12 schedule under ambient room lighting at an ambient temperature of 21°C to 23°C and 50% to 70% humidity. For irradiation treatment, a cesium-137 radiation source emitting γ-rays at a rate of 0.82 Gy/min was used. Twenty-four wild-type and 27 $Cry1^{-/-}Cry2^{-/-}$ mice were treated at 8 weeks of age with a single dose of 4 Gy at zeitgeber time (ZT) 10. (By convention, ZT0 is the time of lights-on and ZT12 is the time of lights-off).

Fibroblast cell lines, growth rate measurement, and UV and ionizing radiation survival. Dermal fibroblast cell lines were isolated as described (18) using skin biopsies from wild-type and $Cry1^{-/-}Cry2^{-/-}$ mice. Fibroblasts underwent spontaneous immortalization.

Cells were grown in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gemini, Woodland, CA) and 100 units/mL penicillin and 100 μg/mL streptomycin (Life Technologies). Cells were maintained in an incubator at 37°C under 5% CO_2. For growth rate measurements, cells were plated in 150-mm plates at low density to ensure continued proliferation throughout the duration of the experiment. Cells were trypsinized and counted at the indicated time points using a hemocytometer.

Cell survival to radiation was determined by clonogenic assay. Wild-type and $Cry1^{-/-}Cry2^{-/-}$ fibroblasts were plated at low density to ensure the formation of ~200 colonies per 100-mm plate in the absence of radiation treatment. Following plating, cells were incubated in growth medium for 10 to 14 hours and treated with either UV or ionizing radiation of appropriate

doses. UV treatment at the indicated doses was done using a GE germicidal lamp emitting mainly at 254 nm. Cells were washed with PBS, irradiated with UV at a fluence rate of 0.65 J/m^2s in the absence of growth medium, and new growth medium was added after treatment. For ionizing radiation treatment, cells in growth medium were irradiated from a cesium-137 radiation source at a rate of 0.82 Gy/min. After radiation treatment, cells were incubated for 9 to 10 days until colonies were readily visible. Cells were fixed for 20 minutes in 3:1 methanol/acetic acid, rinsed with water, and stained with Giemsa stain. Colonies containing >50 cells were scored.

Flow cytometry. Fibroblasts were grown in DMEM and plated to achieve a density of one to two million cells at the time of experiment. Cells were treated with ionizing radiation as described above. At the indicated time posttreatment, cells were trypsinized and fixed in 70% ethanol. DNA content analysis was done using propidium iodide staining and a Beckton Dickinson FACScan analytic flow cytometer. Data acquisition and representation was done with Cicero Software (Cytomation, Inc., Fort Collins, CO).

Western blotting. Standard Western blotting procedures were used for Wee1, c-Myc, and Bmal1 proteins from wild-type and Cry mutant mouse liver extracts and fibroblast cell lysates. For some of the Western blots, we used livers from Cry mutant mice in an *rd/rd* background because of the ready availability of these animals (18). The *rd* mutation does not affect the molecular clock (8, 9). Anti-Wee1 rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cMyc mouse monoclonal antibody (9E10, Santa Cruz Biotechnology) were used to probe for Wee1 and c-Myc, respectively. Anti-Bmal1 guinea pig polyclonal antibody (19) was a kind gift of Dr. Choogon Lee (Florida State University). Anti-IgG rabbit and mouse antibodies (Amersham Biosciences Group, Piscataway, NJ) and anti-IgG guinea pig antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used for secondary antibody blotting.

Results

Expression of Bmal1, Wee1, and c-Myc in cryptochromeless mice and fibroblasts. In the conventional clock model, Clock and Bmal1 constitute the positive regulatory branch, whereas the Crys and Pers make up the negative branch of the autoregulatory

oscillatory circuit (Fig. 1). In addition, Crys and Pers seem to stimulate transcription of Bmal1 by an ill-defined mechanism (8, 9). Consequently, Per and Cry mutants often exhibit similar molecular and behavioral phenotypes. However, it must be noted that there are subtle but significant differences between Cry and Per mutant mice. Hence, it is not possible to predict the responses of Cry or Per mutant cells and mice to a certain treatment based on responses observed with their Per or Cry mutant counterparts, respectively.

Recently, it was reported that in Cry mutant mice, the level of antimitotic Wee1 kinase was elevated in the liver (12) and that in Per2 mutant mice, *c-myc* transcription was up-regulated (11). Both genes are CCGs that contain multiple E-boxes in their promoters, which are recognized by the Clock-Bmal1 complex. In the case of *wee1*, the binding of Clock-Bmal1 to the promoter stimulates transcription; in the case of *c-myc*, binding of the heterodimer (or of the NPas2-Bmal1 complex) inhibits transcription. The elevation of Wee1 in the Cry mutant was ascribed to the lack of inhibition of Clock-Bmal1 by cryptochrome (12, 20). Up-regulation of *c-myc* transcription in the Per2 mutant was ascribed to the reduced level of Bmal1 because Per2, in addition to its inhibitory effect on the Clock-Bmal1 complex, stimulates transcription of the *Bmal1* gene (11, 21, 22). Thus, to begin to investigate the effect of cryptochrome knockout on cellular and organismic response to DNA damage, we wished first to determine the expression of Bmal1, c-Myc, and Wee1 in Cry mutant mice and fibroblasts.

Bmal1 is expressed very highly and at comparable levels in wild-type and Cry mutant fibroblasts (Fig. 2A). However, in comparing Bmal1 expression in the liver, we find that Bmal1 is expressed with a circadian periodicity in wild-type liver but at a reduced and nonoscillating level in the Cry mutant (Fig. 2B), in agreement with a previous report (19). The level of c-Myc has not been previously analyzed in Cry mutant mice. However, it was reported that *c-Myc* transcription is up-regulated in Per2 mutant mice, presumably due to a reduced Bmal1 level (11). Because Bmal1 is reduced in the Cry mutant, we expected to observe elevated c-Myc levels in Cry mutant mice and possibly fibroblasts as well. Figure 2C-D shows that c-Myc is expressed in mutant and wild-type fibroblasts at comparable levels and at statistically indistinguishable and non-oscillating levels in the livers of mutant and wild-type mice. These results differ from those of the previous study, which reported that reduction in Bmal1 levels as a consequence of Per2 mutation causes a substantial increase in c-Myc activity (11). However, in that study the c-Myc RNA but not protein level was measured. Regardless of the cause of the discrepancy between the two studies, it seems that a decrease in the Bmal1 transcriptional regulator does not necessarily lead to increased c-Myc protein in the mouse liver or fibroblasts.

Because it has been reported that Cry and Per regulate *Wee1* (12) and *c-Myc* transcription through their effects on Bmal1 activity and Bmal1 transcription, respectively, we reasoned that the reduced Bmal1 in the Cry mutant would be accompanied by elevated Wee1. Figure 3A-B shows that Wee1 is indeed elevated in Cry mutant fibroblasts and liver, respectively, relative to the wild-type control and the moderately elevated (relative to the low level of Wee1 in wild-type mice) Wee1 in Cry mutant liver does not oscillate, in agreement with previous reports (12, 20).

DNA damage checkpoints and DNA repair in cryptochrome-less fibroblasts. Wee1 is a cell cycle kinase that plays a key role in
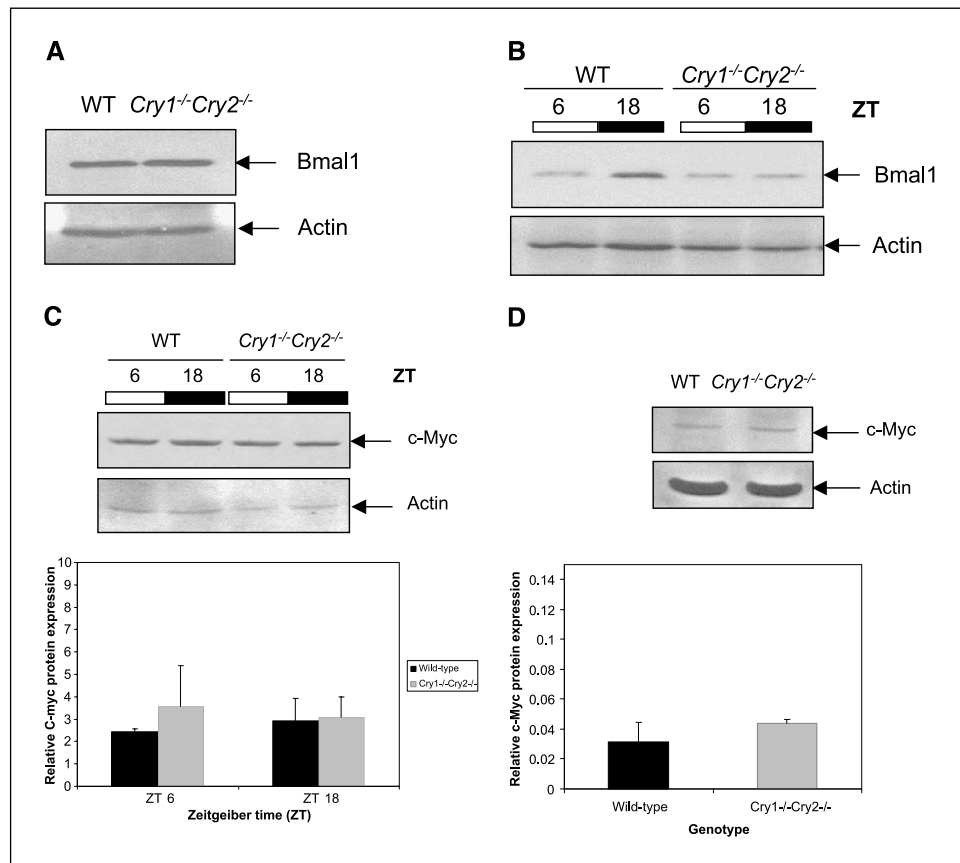
Figure 2. Effect of cryptochrome on Bmal1 and c-Myc expression in mouse fibroblasts and mouse liver. *A* and *B,* Bmal1 expression analysis. *A,* (200 μg) from wild-type (*WT*) or Cry mutant fibroblasts were analyzed for Bmal1 expression by Western blotting using actin as a loading control. *B,* liver extracts from wild-type (*WT*) and Cry mutant mice were prepared at the indicated zeitgeber times (ZT0 = lights on, ZT12 = lights off) and analyzed for Bmal1 expression using actin as a loading control. It has been reported that in the liver of wild-type mice, Bmal1 levels are lowest at ZT6 and highest at ZT18. *C* and *D,* c-Myc expression analysis. Extracts (200 μg) from fibroblasts (*A*) or mouse liver (*B*) were probed for Wee1 and actin by Western blotting. *Top,* Western blot; *bottom,* quantitative analysis of Western blot. Averages of three independent experiments, including the one shown in *top. Columns,* average (*n* = 3); *bars,* SD.
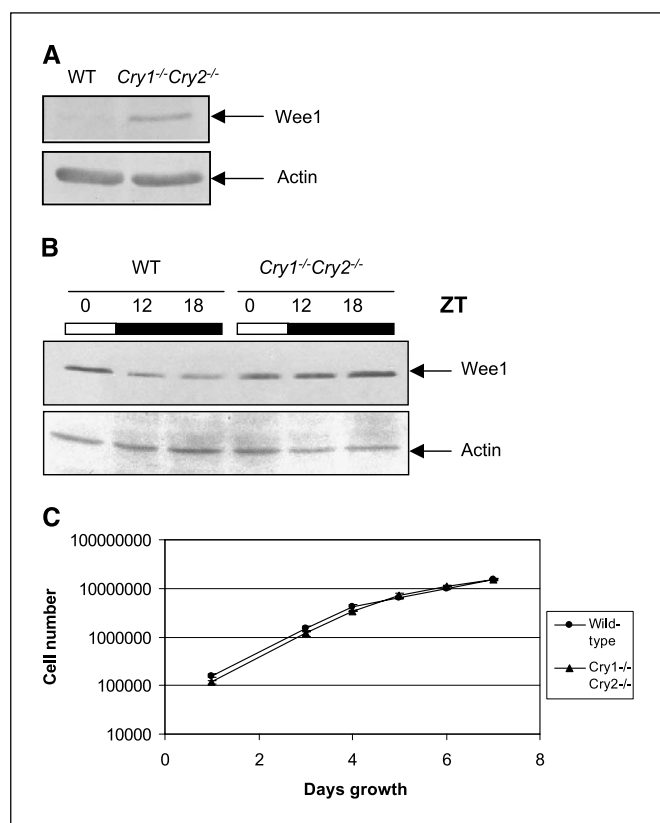
Figure 3. Effect of cryptochrome on Wee1 expression and growth rate of mouse fibroblasts. *A* and *B,* Wee1 expression analysis. Extracts (200 μg) from fibroblasts (*A*) or mouse liver (*B*) were probed for Wee1 and actin by Western blotting. Liver extracts were prepared at the indicated zeitgeber times. *C,* growth kinetics of immortalized fibroblasts from wild-type (●) and $Cry1^{-/-}Cry2^{-/-}$ (▲). Cells were plated at densities of ~ 10^4 cells per dish and cells were counted at the indicated time points. *Points,* n = 3; *bars,* SD.

requisite sensitivity to detect a 15% difference in growth rate. Regardless of these considerations, however, it is safe to conclude that the ~ 3-fold increase in Wee1 level in cryptochromeless cells does not cause a comparable decrease in cell growth rate and it has no detectable effect on the DNA damage checkpoint response.

As a further test of DNA damage checkpoint response in the absence of a functional clock, we determined the survival of cryptochromeless fibroblasts to UV and ionizing radiation. Figure 4 shows that the Cry mutant is indistinguishable from wild type in its response to both genotoxicants. As most checkpoint defects increase cellular sensitivity to genotoxicants (23, 24) these results further confirm the conclusion based on Table 1 that in the absence of cryptochrome, DNA damage checkpoints are normal. Of equal significance, the data in Fig. 4 also reveals that nucleotide excision repair (as measured by UV resistance) and double-strand break repair (as measured by resistance to ionizing radiation) are unaffected by loss of the clock. Thus, we conclude that, in the absence of a circadian clock, both DNA damage checkpoints and the two major DNA repair pathways for repairing base damage and backbone breaks operate essentially normally.

Ionizing radiation–induced morbidity and mortality in cryptochromeless/clockless mice. Per2 mutant mice lack circadian rhythm and were reported to be more sensitive to the acute and chronic effects of ionizing radiation relative to wild-type controls (11). The acute effects included hair graying, hair loss, and skin ulcerations. Chronic effects were an ~ 10-fold increase in incidence of lymphomas relative to wild-type controls and a comparable increase in mortality within 70 weeks following treatment with 4 Gy of ionizing radiation. The increased incidence of lymphomas and higher mortality rate were ascribed in part to the elevated c-Myc and in part to a general dysregulation of cell cycle genes as a result of clock disruption.
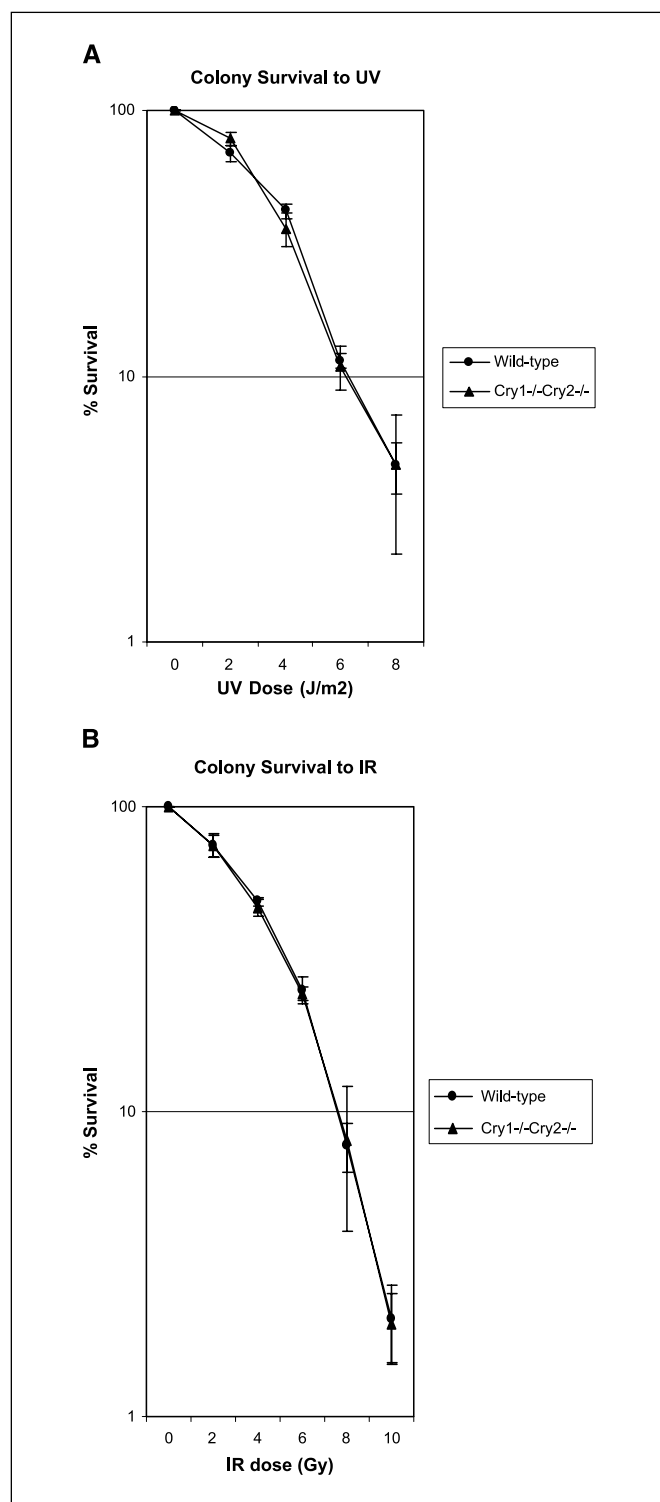
To determine whether or not clock disruption by any means has similar effects on ionizing radiation–induced morbidity and mortality, we irradiated 8-week-old $Cry1^{-/-}Cry2^{-/-}$ mice and

the G$_2$-M transition. Ongoing DNA replication or the presence of DNA damage activate Wee1, which then phosphorylates Y14 of the Cdc2 mitotic kinase, causing its inactivation and delay of mitosis or arrest of the cell cycle at the G$_2$-M interface (23, 24). It is conceivable that elevated Wee1 in Cry mutant mice phosphorylates Cdc2 at an increased rate even in nonstressed cells, slowing down the G$_2$-M transition and the overall growth rate. Indeed, it was reported that 3 days after partial hepatectomy, the regenerating liver of cryptochromeless mice contained fewer mitotic figures and lagged in weight recovery by about 15% relative to the wild-type control (12). Based on these considerations, then, we expected cryptochromeless fibroblasts to grow more slowly than wild-type fibroblasts and to arrest for a longer period at the G$_2$-M boundary after DNA damage, possibly remaining at this interface indefinitely and thus undergoing replicative death. Interestingly, we did not observe either of these two effects. First, cryptochromeless fibroblasts grew at a rate indistinguishable from wild-type fibroblasts under standard growth conditions (Fig. 3*C*). Second, and more importantly, after 8 Gy of ionizing radiation, the cryptochromeless fibroblasts seem to arrest at G$_2$-M and to recover from this arrest at the same rate as wild-type fibroblasts (Table 1). It is likely that multiple factors contribute to the difference between the reported effect of cryptochrome on liver regeneration rate and our results with tissue culture. It is also possible that the tissue culture conditions do not possess the

Table 1. G$_2$-M checkpoint in wild-type and Cry mutant fibroblasts after ionizing radiation treatments

Genotype	Time (h) post-IR (8 Gy)	% Cell population		
		G$_1$	S	G$_2$-M
Wild-type	0	45.16	30.28	**25.98**
	4	31.53	36.24	34.08
	8	14.25	31.41	**56.06**
	12	18.09	16.85	65.72
	24	39.71	27.95	**33.68**
$Cry1^{-/-}Cry2^{-/-}$	0	41.20	34.34	**26.35**
	4	24.17	42.43	36.37
	8	10.64	36.63	**56.50**
	12	21.04	9.91	68.05
	24	39.08	26.88	**32.94**

NOTE: Exponentially growing cells were irradiated and at the indicated times cells were fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content. The fraction of cells at the G$_2$-M boundary at 0, 8, and 24 h are shown in bold to highlight the G$_2$-M arrest and subsequent recovery.
Abbreviation: IR, ionizing radiation.

A

Colony Survival to UV

y-axis: % Survival (1, 10, 100)
x-axis: UV Dose (J/m2) (0, 2, 4, 6, 8)

Legend:
- ● Wild-type
- ▲ Cry1-/-Cry2-/-

B

Colony Survival to IR

y-axis: % Survival (1, 10, 100)
x-axis: IR dose (Gy) (0, 2, 4, 6, 8, 10)

Legend:
- ● Wild-type
- ▲ Cry1-/-Cry2-/-

Figure 4. Radiation survival of wild-type and cryptochrome mutant fibroblasts. *A,* UV survival; *B,* ionizing radiation (*IR*) survival. ●, wild-type; ▲, Cry mutant. *Points,* averages of three independent experiments (*n* = 3); *bars,* SD.

wild-type controls with 4 Gy of ionizing radiation at ZT10 and followed their survival for 90 weeks. The results obtained differed from those obtained with Per2 mutant mice. First, we did not observe a difference in the timing and intensity of hair graying and loss between Cry mutant and wild-type mice (data not shown).

Second, and most significantly, over the 90-week observation period there was no significant difference in the mortality of irradiated Cry mutant mice and wild-type mice (Fig. 5). Moreover, in contrast to the similarly treated Per2 mutant mice, we did not detect overt lymphomas in irradiated Cry mutant animals. The irradiated mice died from a variety of causes including genitourinary prolapses and infections, paralysis, and seizures that necessitated euthanasia, and in some cases from indeterminable causes. Importantly, however, there was no detectable difference between the causes of death of cryptochromeless and wild-type animals. These results suggest that clock disruption per se does not make mice hypersensitive to the acute effects of ionizing radiation nor does it predispose them to increased incidence of spontaneous or ionizing radiation–induced cancers or mortality from any other cause. The significance of these findings is discussed below.

Discussion

Circadian cycle and cell cycle. The circadian cycle and cell cycle are two global regulatory mechanisms that directly or indirectly influence all biochemical reactions in cells. Hence, it is logical to assume that disruption of one would cause dysregulation of the other with adverse consequences for the cell (25–27). Recent advances in understanding the circadian clock at the molecular level have provided the opportunity to approach the circadian cycle cell cycle connection from a mechanistic perspective. Thus, it was reported that the core circadian clock machinery affects cell cycle progression in proliferating cells by controlling the expression of Wee1, a kinase that regulates the activity of Cdc2 and hence the G_2-M transition (12). Another study concluded that Per2 mutant mice had constitutively elevated levels of expression of the cell growth/proliferation gene *c-Myc* and reduced expression of p53, which plays a critical role in the G_1-S checkpoint (11). Finally, we have recently found that the mammalian Tim plays a direct role in cell cycle checkpoints including the intra-S and replication checkpoints (15). Although the role of the mammalian Tim in the circadian clock is a matter of some debate (13, 14), these studies in
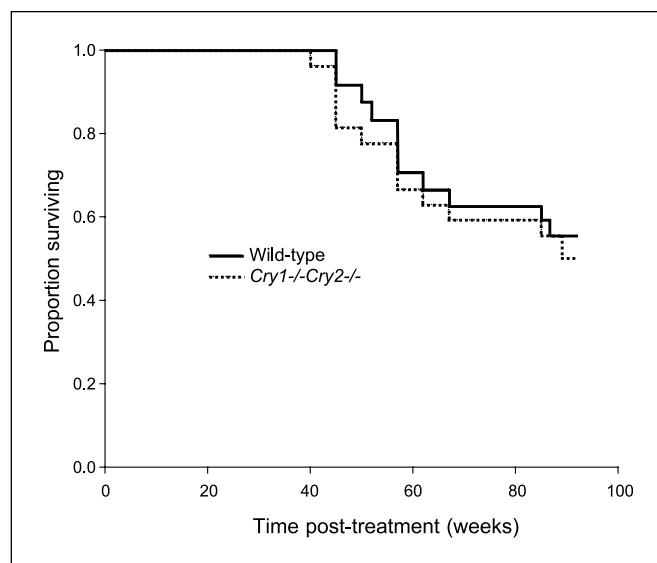
Figure 5. Ionizing radiation survival of wild-type (*solid line*) and Cry1^{−/−} Cry2^{−/−} (*broken line*) mice. Eight-week-old mice were exposed to 4 Gy at ZT10 and were observed for 90 weeks. Survival is plotted according to the Kaplan-Meier method.

aggregate have given credence to the general view that circadian disruption by whatever means may lead to a failure of cell cycle checkpoints (28). However, this view needs to be critically tested experimentally. In particular, it remains to be determined if proteins that have been presumed to be exclusively clock proteins such as Clock and Bmal1, which work as partners in the positive branch of the clock, and Cry and Per, which work as partners in the negative branch, also perform functions outside of the clock and unique to each protein. In the case of mammalian Tim, it is clear that the protein participates in cell cycle control independent of its role in the clock (15). If the so-called canonical clock proteins Clock, Bmal1, Cry, and Per have unique cellular functions outside of the clock machinery, then the mutants of these proteins are expected to exhibit some unique features as well. An additional possibility is that different clock mutants disturb the circadian cycle in different ways, leaving a different pattern of circadian genes "on" and "off" depending on when the clock stops. Although the molecular clock does not "stop" at a given time but rather falls into a stable equilibrium that terminates the cycles in the abundance of its components, one can imagine that leaving certain circadian genes "on" or "off" might cause a susceptibility to cancer or not, without necessarily making cancer susceptibility caused by clock disruption specific to a particular clock gene. Moreover, cell cycle and carcinogenic transformation are also regulated by homeostatic mechanisms and therefore a potentially cell cycle–disruptive effect of a clock gene mutation might be mitigated or completely alleviated by such compensatory mechanisms. Consequently, a priori it cannot be known whether all circadian disruptions will have the same effect on cell cycle and DNA damage checkpoints and whether their potential cell cycle-disruptive effects will necessarily lead to actual disruption.

Cell cycle checkpoints in the absence of circadian rhythm. In this study, we analyzed the growth properties, cell cycle checkpoints, and DNA repair capacity of $Cry1^{-/-}Cry2^{-/-}$ fibroblasts and the susceptibility to ionizing radiation–induced cancer and mortality of $Cry1^{-/-}Cry2^{-/-}$ mice. Based on published reports of Cry and Per mutations on cellular growth (12) and damage response (11), we were expecting the Cry mutants to be defective in DNA damage checkpoints and to exhibit increased ionizing radiation–induced morbidity and mortality for the reasons outlined below.

First, current clock models presume that Cry and Per function as heterodimers and because it has been reported that in Per2 mutant mice, *c-Myc* is up-regulated, p53 is down-regulated, and there is a general cell cycle dysregulation (11), we expected that the Cry mutant fibroblasts would exhibit some cell cycle checkpoint defects and that the Cry mutant mice, like the Per2 mutants, would be cancer prone. We find that in Cry mutant livers, Bmal1 expression, as in the case of the Per2 mutant, is reduced. However, in an apparent contrast to the Per2 mutant mice, the *c-Myc* level is not elevated in either Cry fibroblasts or mice, indicating that Pers and Crys affect c-Myc expression differently. It must be noted, however, that in the Per2 mutant mice the *c-Myc* RNA but not the protein level was measured. It is conceivable that even in the Per2 mutant mice the elevated level of *c-Myc* mRNA is not accompanied by elevated c-Myc protein and that the increased incidence of spontaneous and ionizing radiation–induced lymphomas reported in these animals was caused by an unknown effect of Per2 on cell growth and proliferation.

Second, it was reported (12) that Wee1 kinase, which inhibits the G_2-M transition, is elevated in Cry mutant mice and evidence was presented suggesting that after partial hepatectomy, the liver of Cry mutant mice regenerates more slowly than that of wild-type controls, presumably because of inhibition of mitosis by elevated Wee1. In agreement with previous reports (12, 20), we find that Wee1 is elevated in Cry mutant fibroblasts and liver and other tissues of Cry mutant mice. However, despite this elevation in Wee1 level the Cry mutant fibroblasts grow at a rate indistinguishable from the wild-type controls. It seems that exponentially growing cultures of mutant fibroblasts had fewer mitotic figures than wild-type (data not shown). We assume that a slight delay in mitotic entry was compensated by faster progression through other phases of the cell cycle such that there was no change in overall growth rate relative to the control. Importantly, the mutant cells did not exhibit an amplified checkpoint response to DNA damage and, consequently, their kinetics of checkpoint-induced inhibition of cell cycle progression through G_2-M was indistinguishable from wild-type controls. This again indicates the presence of compensatory mechanisms that ensure normal checkpoint response even in the presence of elevated Wee1. Our results seem contradictory to the report indicating slower recovery of liver mass in Cry mutant mice after partial hepatectomy (12). However, it is possible that the apparent discrepancy may stem from differences in stress responses induced by DNA damaging agents, as opposed to partial hepatectomy, and the nature of the cell types analyzed in the two studies.

Circadian disruption and cancer predisposition. Epidemiologic studies have suggested that circadian disruption may contribute to cancer incidence (1) and adversely affect the course of the disease (2). A prospective study with Per2 mutant mice seems to have provided a molecular explanation for the connection between circadian rhythm disruption and cancer predisposition. Our work indicates that disruption of the clock does not necessarily predispose mice to cancer. The cancer predisposition of Per2 mutant mice was ascribed, in large part, to decreased Bmal1 expression and the consequent increase in c-Myc expression. Bmal1 expression is reduced in both Per2 and Cry mutant mice (ref. 19; this work); hence, it remains to be proven that the increased c-Myc transcription reported in Per2 mutant mice is a direct consequence of Bmal1 reduction, which, in the form of either Bmal1-NPas2 or Bmal1-Clock heterodimer, represses *c-Myc* transcription (11). Whatever the cause of elevation of *c-Myc* transcription in Per2 mutants, we do not observe a measurable change in the c-Myc protein level in Cry mutant mice; hence, it is possible that the absence of Per2 makes mice cancer-prone not by overexpression of c-Myc but through an unknown mechanism. It must be noted, however, that the ionizing radiation–induced mortality of the wild-type mice in our study was the same as that of the Cry mutant and, importantly, it was significantly higher than that of the wild-type control mice used in the Per2 mutant mouse study. It is possible that the genetic background (C57BL/6J in our study and C57/SV129 in the Per2 study) affects the susceptibility of even "wild-type" mice to both ionizing radiation–induced cancers and ionizing radiation–induced mortality. In our study, both the Cry mutant and the wild-type control mice were in C57BL/6J background; therefore, we suggest that the lack of difference in morbidity and mortality between the wild-type and Cry mutant mice is most likely because circadian clock disruption by eliminating Cry does not affect cell cycle checkpoints, DNA repair, or apoptosis in a way that would result in increased mutations, reduced apoptosis, and eventually cancer.

It should be of interest to find out how mutations in other clock genes, in particular *Clock* and *Bmal1*, affect the incidence of spontaneous and ionizing radiation–induced cancers. While this article was in preparation, a report was published showing that the sensitivity of mice to the acute effects (weight loss and death) of high doses of cyclophosphamide, an alkylating anticancer drug, was strongly dependent on the circadian time of drug delivery (29) and that the Cry mutant used in our study was resistant to the acute effects of cyclophosphamide at all times of the day. Clearly, further studies are needed to explain the apparent resistance of Cry mutant mice to the acute effects of cyclophosphamide. Regardless of the precise mechanism of the resistance, the results of the study on the acute effects of a DNA damaging agent and our study on the long-term effects of ionizing radiation are, in general, in agreement in demonstrating that clock disruption per se does not make mice more susceptible to the acute or chronic effects of DNA-damaging agents.

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